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INTERACTION OF ELECTROMAGNETIC FIELDS WITH CHONDROCYTES IN GEL CULTURE

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NOTICES

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The animals involved in this study were procured, maintained, and used in accordance with the Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources - National Research Council.

The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.

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The research accomplished during this project period focused on control experiments designed to establish whether cartilage cells from normal cartilage will continue to synthesize and accumulate normal extracellular matrix in agarose gel culture. This information is essential to properly design experiments to qualify changes in chondrocyte biosynthesis due to applied electromagnetic fields. The results suggest that both normal chondrocytes and swarm rat chondrosarcoma cells in agarose culture can continue to synthesize matrix macromolecules at a rate similar to or slightly higher than that in normal cartilage; also, that chondrocytes in agarose can successfully mediate assembly and accumulation of normal, mechanically functional extracellular matrix.

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SUMMARY

The research accomplished during this project period has focused on control experiments designed to establish whether cartilage cells extracted from native cartilage will continue to synthesize and accumulate a normal, cartilage-like extracellular matrix in agarose gel culture. This information is essential to properly design experiments whose purpose is to quantify *changes in chondrocyte biosynthesis due to applied electromagnetic fields.*

We have, therefore, characterized the *extent* and the *time evolution* of chondrocyte proliferation, synthesis of glycosaminoglycans (GAG) and proteins, loss of GAG, and total deposition of GAG-containing matrix within agarose gels by quantifying:

(1) Total DNA content of agarose/chondrocyte disks (as a measure of cell number) versus time in culture;

(2) Total GAG content of agarose/chondrocyte disks versus time in culture;

(3) Incorporation of radiolabelled sulfate and proline (as a measure of the rate of biosynthesis of GAG and proteins) at selected times during long-term culture;

(4) Total GAG lost from agarose/chondrocyte disks and released into the medium at selected times during long-term culture.

To assess whether the matrix deposited within the agarose gel is, in fact, a cartilage-like mechanically functional matrix, we measured several important mechanical and electromechanical properties of the agarose/chondrocyte disks at selected times during long-term culture. These same properties characterize, in part, the ability of normal, intact cartilage to function properly in a joint. These properties have been previously measured in normal cartilage by the same means and can, therefore, be directly compared to those of the agarose/chondrocyte disks. These properties include:

(1) Equilibrium elastic modulus;

(2) Hydraulic permeability;

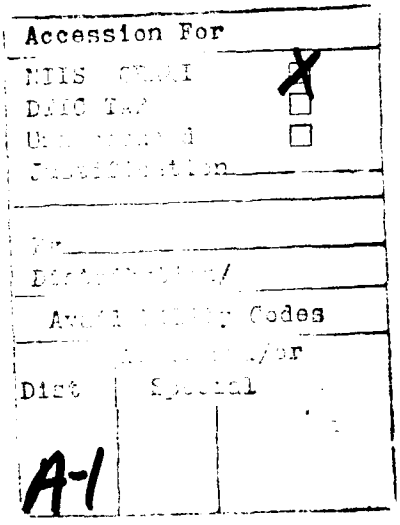
(3) Streaming potential induced by oscillatory mechanical compression;

(4) Dynamic stiffness in sinusoidal mechanical compression.

The results of these control studies, described in detail next, suggest that **(1) both normal chondrocytes and Swarm rat chondrosarcoma cells in agarose**

culture *can*, under proper culture conditions, continue to synthesize matrix macromolecules at a rate similar to or slightly higher than that in native cartilage, and (2) chondrocytes in agarose can successfully mediate the assembly and accumulation of a normal, mechanically functional extracellular matrix.

These results provide support for the long-term objectives of this research concerning the effects of electromagnetic fields. Thus, the chondrocyte/agarose system appears to be an appropriate model for the study of mechanisms of interaction of electromagnetic fields with cells. Here, the cells can be maintained in a natural environment which, in certain ways, is more amenable than that of native cartilage to the study of mechanism at the single cell level.



INTERACTION OF ELECTROMAGNETIC FIELDS WITH CHONDROCYTES IN GEL CULTURE

INTRODUCTION

The interaction of electromagnetic fields with biological tissues is of increasing importance from the standpoint of potential health hazards and of possible beneficial (e.g., diagnostic and therapeutic) effects. While much of the literature to date has focused on experimental observation and verification of putative field effects, increasing attention is now being paid to the physical mechanisms that may underlie such electromagnetic interactions. There is still little understanding of basic physical mechanisms or of the biochemical pathways whose disturbance is essential for interaction to occur.

Electromagnetic interactions with biological tissues may involve several different physical and biochemical processes. Sufficiently intense radiation can result in significant damage to cells by thermal processes alone [1]. The effects of subthermal processes have been harder to quantify. The long-term goal of our research is aimed at identifying the mechanisms of normal cell functioning that are altered by electromagnetic fields.

To address these issues, we have initiated a project that focuses on the biosynthesis of highly specialized protein and polysaccharide molecules by mammalian connective tissue cells, with and without exposure to the combined effects of electrical and chemical perturbations. Since the synthesis of these macromolecules is one of the most important biological functions of connective tissue cells, altered biosynthetic response is known to be a most sensitive indicator of the effectiveness of an environmental stimulus, and of the pathway by which the stimulus evokes a response.

In particular, we have studied the synthesis of highly charged proteoglycan (PG) molecules and their glycosaminoglycan (GAG) constituents, collagens, and other noncollagenous proteins by normal cartilage cells (chondrocytes) extracted from articular cartilage, and in rat chondrosarcoma cells (a continuous cell line). The overt gene expression of chondrocytes results in the synthesis and deposition of a structurally integrated extracellular matrix composed of these macromolecules [2]. The use of cartilage cells is motivated, in part, by the fact that these cells are naturally subjected to endogenous electric fields over a 5-6 decade frequency range at current densities as high as 1 mA/cm^2, produced by *in vivo* mechanical loading [3,4].

To examine these biosynthetic responses, it is essential to use cell populations that are as homogeneous as possible. Because cartilage cells vary with depth in the tissue

and along a joint surface, it is advantageous to either extract homogeneous subpopulations or to uniformly mix all extracted cells from the tissue, and culture them in a medium in which applied field parameters can be precisely controlled. For this and other important reasons, we have chosen to culture and test the cells in agarose gel discs. Such culture techniques have been well-established for normal chondrocytes and chondrosarcoma cells. In addition, the chondrocyte-containing agarose gels are transparent. Therefore, an array of assays will ultimately enable additional characterization of single cell responses, including staining and cytofluorescence techniques that have already been developed elsewhere.

In addition, the agarose/cell system will more easily enable us to couple our experimental methods and results to the theoretical models of interaction mechanisms recently developed in our laboratory [5-9] and by other investigators [10]. For example, electrodiffusion and diffusion reaction processes in an ionized extracellular matrix [7-9] may lead to field-induced changes in the concentrations of certain ions, nutrients, and cell products. The agarose gel constitutes a medium whose charge density and chemical composition can be altered in a well-defined manner allowing us to test the influence of these mechanisms on cell-field interactions. In addition, changes in transmembrane potential have been postulated to be an important consequence of applied fields in certain frequency ranges. The agarose gel system may enable fluorescence detection of changes in transmembrane potential in real time, as the amplitude and frequency of the applied field is swept. To compare and interpret experimental results in terms of theoretical models of the above mechanisms, direct current frequencies of 10-100 MHz are of interest.

To accomplish the long-term goals of this research, the specific objectives of the initial 6-month effort under Task 0003 of Contract F33615-87-D-0626 were:

(1) Adapt the methodology for agarose gel culture of chondrocytes to enable preparation of cylindrical disk and slab specimens containing cells at the appropriate density suitable for exposure to direct current frequencies of 10-100 MHz.

(2) Quantify cellular synthesis of proteoglycans, collagens, and other proteins in control agarose gels in which the fields are not applied, using normal chondrocytes extracted from calf articular cartilage and chondrosarcoma cells (a homogeneous, continuous cell line).

(3) Begin to assemble facilities and fabricate specialized chambers for applying to electric fields corresponding to current densities over a wide range of amplitude and frequencies.

BACKGROUND AND OVERVIEW

Articular cartilage is the dense, skeletal connective tissue that functions as a bearing material in synovial joints. Cartilage from adult animals and humans is

avascular, aneural, and alymphatic. The tissue's sparse population of cells (chondrocytes) derives its nutrition primarily from the synovial fluid in the joint cavity [11]. The chondrocytes are responsible for the synthesis, maintenance, and gradual turnover of an extracellular matrix composed primarily of hydrated type II collagen fibrils and highly charged proteoglycan molecules [12] which together account for 20-30% of tissue wet weight. These matrix macromolecules are shown schematically in Figure 1. The proteoglycan constituents contain a high net charge at physiolcgical pH and thereby exert significant electrostatic swelling forces that help to maintain tissue hydration and help to resist mechanical compression. The hydrated matrix and the tissue's high water content (70-80% of wet weight) are responsible for the complex rheological behavior that characterizes its response to mechanical loads *in vivo*. The structure and biosynthesis of proteoglycans and collagen from human and animal cartilages have been extensively studied during the past decade [13].

When cartilage is statically compressed under physiological conditions, its water content decreases and its fixed charge density increases. This will increase the concentration of all mobile counterion species (e.g., Na^+, H^+) within the matrix and decrease co-ion concentrations (e.g., Cl^-), consistent with the laws of Donnan equilibrium and electroneutrality [14,15]. Investigators have found that such changes in cartilage charge density and hydration result in concomitant changes in the rate of synthesis of proteoglycans and proteins in organ culture explants [16,17]; here, the mechanism appears to be associated with the changes in intratissue (extracellular) pH produced by static compression of the intact tissue [18].

Dynamic compression of cartilage under physiological conditions also produces pressure gradients and fluid flow within the extracellular matrix. *This fluid flow generates electrical potentials and currents known as streaming potentials and currents.* The past few years have seen significant progress in: (1) the verification that an electrokinetic mechanism is responsible for these endogenous electrical signals in cartilage [3,4]; (2) the demonstration that fluid convection of ions past the carboxyl and sulfate fixed charge groups of the proteoglycans are responsible for streaming currents [19]; and (3) the quantification of the amplitude and frequency response of streaming potentials and streaming currents produced in living cartilage organ culture explants by physiological levels of oscillatory mechanical compression [5].

Cartilage is now seen to be one of the most electrically active tissues that does not contain excitable cells. The possibility that endogenous electrical currents generated by mechanical loads may play a role in cellular response has been hypothesized in soft and hard connective tissues [20]. In order to study this important possibility in cartilage, investigators have stressed the importance of quantifying the effects of applied fields on the synthesis of proteoglycans and collagen [20]. Thus, it is well accepted that the *quantity and quality of the proteoglycans and proteins synthesized by chondrocytes* are crucial and sensitive markers of the ability of an environmental stimulus to affect chondrocyte behavior.

Our previous work on the response of mammalian cells to electromagnetic fields has focused on the effect of applied electric currents on the synthesis of stress response and other proteins in intact organ culture explants of cartilage. The results to date show that current densities below 30 mA/cm^2 at frequencies less than 1 kHz *do not* appear to stimulate a stress response. However, total protein synthesis *does* appear to increase with increasing applied current density (Fig. 2) [21].

The fact that the total protein synthesis only appears to increase with relatively high current densities may be due to the heterogeneity of the cell population within the cartilage explant. It is known that cell morphology and biosynthetic response vary with position in the tissue: cells near the upper articular surface are different from those of the middle and deep zones of cartilage [22]. Isolation of the cells from these distinct zones would yield much more homogeneous populations. For this reason, researchers in cartilage biology and biochemistry have recently developed techniques for the sectioning and isolation of specific, homogeneous subpopulations of cartilage cells [22]. Culturing of these subpopulations in *agarose gel* has been found to preserve their distinct morphological [22] and biosynthetic properties [22]. In addition, several groups have used a continuous cell line (Swarm rat chondrosarcoma) for biosynthesis studies, because of the demonstrated homogeneity of the response. Sun et al. [23] have found that the proteoglycans and collagens synthesized by these chondrosarcoma cells are strikingly similar to those of native cartilage.

A major point of departure of the present research is the use of agarose gel culture. This technique is consistent with the long-term objectives and constraints of this project: (a) agarose culture has already been proven to be viable for cartilage cells; (b) cylindrical gel disks containing cells can be constructed, so that the amplitudes of the applied electromagnetic fields can be precisely controlled and determined; (c) the agarose culture technique should enable us to study more homogeneous populations of cells compared to that of intact cartilage explants, and thereby improve our "signal-to-noise ratio"; and (d) because the cell-containing agarose gels are transparent, several techniques will be available for examining single cell responses.

Finally, the agarose/chondrocyte system may also have important applications in the area of connective tissue health and disease, as well as the area of electrical interactions in membrane transport. First, the understanding and possible control of protein and proteoglycan synthesis by cartilage cells is essential in the study of degenerative joint disease (osteoarthritis); agarose gel culture has just begun to be used in the study of cell synthesis of enzymes known to degrade the cartilage matrix in osteoarthritis [22]. Second, our work on electrodiffusion and membrane transport [5-9] should be aided by the ability to study single-cell layers in agarose gel.

METHODS

Articular cartilage from the femoropatellar groove from 1- to 2-week-old calves was harvested as previously described [17] and incubated in DMEM supplemented

with 12.5 mM Hepes, 0.1 mM non-essential amino acids, 0.4 mM L-proline, 10% FBS, 50μg/ml ascorbate, and 0.1% penicillin/streptomycin changed daily at 5% CO_2, 37°C. One week later cells were extracted by sequential pronase and collagenase digestion [24]. In addition, Swarm rat chondrocytes were obtained from Dr. J. Kimura (Rush Presbyterian St. Luke's Medical Center, Chicago), and prepared for incorporation into agarose gels as described [23].

Chondrocytes were mixed into media containing 2% (w:v) low melting temperature FMC SeaPlaque agarose [22,23] at $\sim 2 \times 10^7$cells/ml and cast at 40°C between slab gel electrophoresis plates separated by 1-mm-thick Teflon spacers. After gelling at 4°C for 2 h, 16-mm-diameter by 1-mm-thick disks were cored from the gel. The chondrocyte/agarose disks were subsequently cultured on top of nylon mesh (to promote nutrient diffusion from below). Media (as above) was changed daily and analyzed for GAG content by dimethylmethylene blue (DMB) dye binding [25]. Control disks without chondrocytes were prepared and maintained in the same manner.

The mechanical and electromechanical properties of chondrocyte/agarose disks and control disks were measured in uniaxial confined compression using the test chamber shown schematically in Figure 3, mounted in a Dynastat mechanical spectrometer. Individual disks were removed from culture at selected intervals and placed between Ag/AgCl electrodes separated by a porous platen, all bathed in PBS (Fig. 3) [3]. A 20% static offset strain was applied and the load recorded until stress relaxation reached equilibrium. This equilibrium load and strain was used to compute the equilibrium elastic confined compression modulus. A 0.5% sinusoidal strain was then superimposed at frequencies between 0.01 and 1 Hz, and the resulting oscillatory load and streaming potential were recorded. Disks were then removed, weighed, lyophilized, and frozen. The hydraulic permeability, electrokinetic coefficient, oscillatory stiffness and streaming potential, were computed from the data as described previously [3,4].

Each week, groups of 9 to 12 3-mm-diameter disks were separately incubated for 16 h in media containing 10 μCi/ml [^{35}S]sulfate and 20 μCi/ml [5-^{3}H]proline to assess GAG and protein synthesis, respectively. These disks were washed in DMEM and digested with papain. Radiolabel incorporation was assessed by scintillation counting, and GAG was assessed by the DMB dye binding assay. In addition, the content of DNA was measured in these same specimens by means of a fluorescence enhancement assay using the Hoechst 33258 dye [26]. (See Appendix).

RESULTS AND DISCUSSION

Normal Chondrocytes

Table 1 shows the properties of agarose/chondrocyte disks at day 26 in culture, and compares these properties to those of intact calf articular cartilage similar in age

and location to that from which the cells were extracted. In agarose/chondrocyte disks, total GAG content increased significantly over 4 weeks in agarose culture (Fig. 4) reaching ~25% of that in native parent cartilage disks (Table 1). By day 26, the amount of GAG alone was approximately the same as the agarose content of agarose/chondrocyte disks. DNA content appeared to increase slightly during early weeks (Fig. 4).

Sulfate and proline incorporation (Fig. 4) reflects the rate of synthesis of GAG and total protein, respectively. Sulfate and proline incorporation levels in agarose culture (Fig. 4) were greater than or equal to that in the intact parent cartilage (Table 1). GAG accumulation rate (Table 1), calculated by linear regression of GAG content on days 6, 13, and 20 (Fig. 4), is significantly higher than that in intact cartilage. The rate at which GAG was released from the agarose disks (Fig. 5) was slightly higher than that in normal cartilage (Table 1), although this rate decreased as matrix accumulated in the gel.

Cell-laden disks showed significantly enhanced equilibrium modulus, dynamic stiffness, and streaming potential with increasing time in culture compared to controls (Fig. 6), consistent with the increased GAG content found in the same specimens. The existence of the streaming potential and the increase in the potential with time in culture is especially significant, since this streaming potential gives a nondestructive measure of the quality of the newly synthesized extracellular matrix secreted and assembled by the cells. If the newly synthesized proteoglycans had not been functionally immobilized within the developing matrix (as occurs in intact cartilage), there would have been little or no increase in streaming potential (Fig. 6). In a separate experiment with calf metacarpal phalangeal chondrocytes in agarose, disks at 6 weeks exhibited ~2 fold greater streaming potential (Fig. 7) and dynamic stiffness (Fig. 8) than the day 26 agarose disks in Figure 6.

The frequency response of the streaming potential and dynamic stiffness is also an important measure of the functional behavior of the extracellular matrix. The dynamic stiffness and streaming potential of agarose/chondrocyte disks increased with frequency in a manner characteristic of the poroelastic behavior of cartilage [3,4]. The frequency dependence of the amplitude and phase of the streaming potential of normal cartilage is shown in Figure 9 (data from [4]). The increase in streaming potential amplitude with increasing frequency is due to the increase in relative fluid velocity produced at higher compression rates. This relative fluid velocity is responsible for producing the streaming potential. The phase of the potential and stiffness gives an additional measure of the relative importance of fluid flow and elastic deformation in the compressional behavior of the matrix.

Sinusoidal stress-strain and streaming potential were essentially linear in the 0.01-1 Hz range (total harmonic distortion $< 8\%$). The hydraulic permeability of agarose/chondrocyte disks at day 26 was higher than that of normal cartilage (Table 1), consistent with a larger pore size and lower GAG content as compared to carti-

TABLE 1. SUMMARY OF BIOPHYSICAL AND BIOCHEMICAL PROPERTIES OF AGAROSE/CHONDROCYTE DISKS DURING LONG-TERM CULTURE.

	Chondrocyte/ Agarose disks†	Calf Cartilage 'parent disks'
Total DNA (μg/μl disk vol.)	0.14 ± 0.02 (12)	0.56 ± 0.13 (30) [6]
Cell Density (10^6cell/ml) (7.7pg/cell[7])	20.2 ± 2.8 (12)	73 ± 17 (30) [6]
Total GAG (μg/μl disk vol.)	15.9 ± 1.0 (12)	57 ± 11 (30) [6]
35-S-Incorp. (nmol sulfate/10^6cell/day)	50 ± 13 (12)	45 ± 21 (28) [6]
3H-P-Incorp. (nmol proline/10^6cell/day)	41 ± 10 (12)	20.1 ± 9.5 (28) [6]
GAG release rate (% GAG/day)	1.4 ± 0.2 (12) ✱	1.09 ± 0.04 (12) [8]
GAG accumulation rate (% GAG/day)	5.8 ± 0.3 (12) ✱	3.0 [9]
Equilibrium Modulus (kPa)	31.0 ± 5.9 (3)	470 [10]
Hydraulic Perm. ($m^4/N{\cdot}s \times 10^{15}$)	8.0 ± 3.8 (3)	3.5 [10]
Electrokinetic Coefficient (mV/MPa)	3.87 ± .68 (3)	20
Dynamic Stiffness (0.5Hz) (MPa)	0.95 ± 0.17 (3)	4.7 [10]
Streaming Potential (0.5Hz) (μV/%)	38.4 ± 1.9 (3)	150-600 [10]
† data for day 26 except ✱ = day 13	MEAN ± SD (n)	MEAN ± SD (n)

lage. The cell-laden gels expanded slightly (~5%) over the culture period, most likely due to the swelling pressure of the proteoglycans. Control gels contracted slightly (~5%), which was consistent with a small increase in stiffness observed over the first few weeks.

Swarm Rat Chondrosarcoma Cells

In another set of experiments, Swarm rat chondrosarcoma cells were incorporated into 2% agarose gels as above. During 4 weeks in culture, the total GAG content within the agarose/chondrocyte disks increased almost 20-fold (Fig. 10). DNA content also increased by a lesser amount (Fig. 10). After 4 weeks, dynamic stiffness and streaming potential of cell-laden disks had also increased above control levels (Fig. 11). These experiments were actually performed before those involving normal calf chondrocytes. Because of changes and improvements in the methodology in mixing and casting the gels, it is difficult to compare the absolute value of changes observed with normal chondrocytes and chondrosarcoma cells thus far. It is clear, however, that definitive changes have been observed with both types of cells.

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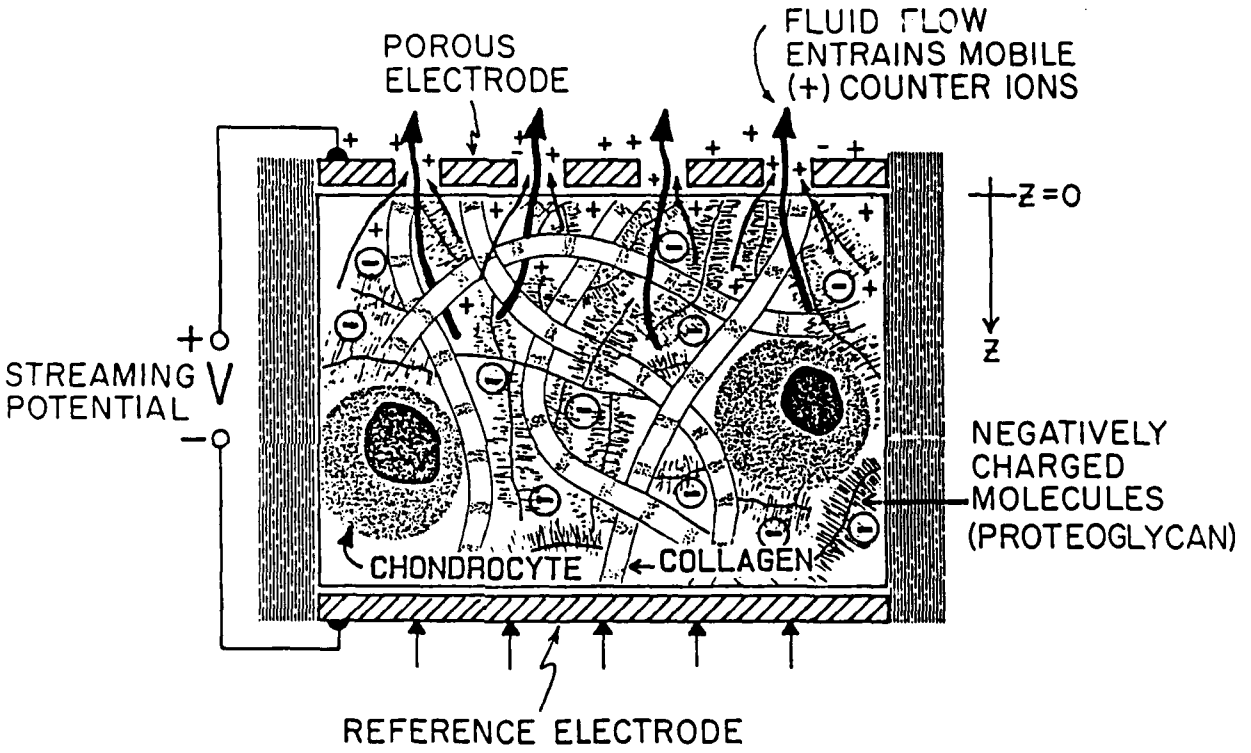

Figure 1. Schematic of cartilage tissue showing cells (chondrocytes) in a highly hydrated extracellular matrix composed primarily of collagen fibrils and proteoglycans, the latter containing fixed negative charge groups. When cartilage is compressed between a bottom impermeable electrode and a porous upper electrode, the upward fluid flow convectively entrains mobile counterions causing a slight separation between mobile and fixed charge. This separation generates a streaming potential difference that can be measured by the electrodes. The resulting streaming current *in vivo* may reach 1 mA/cm^2 during physiological loading of joints.

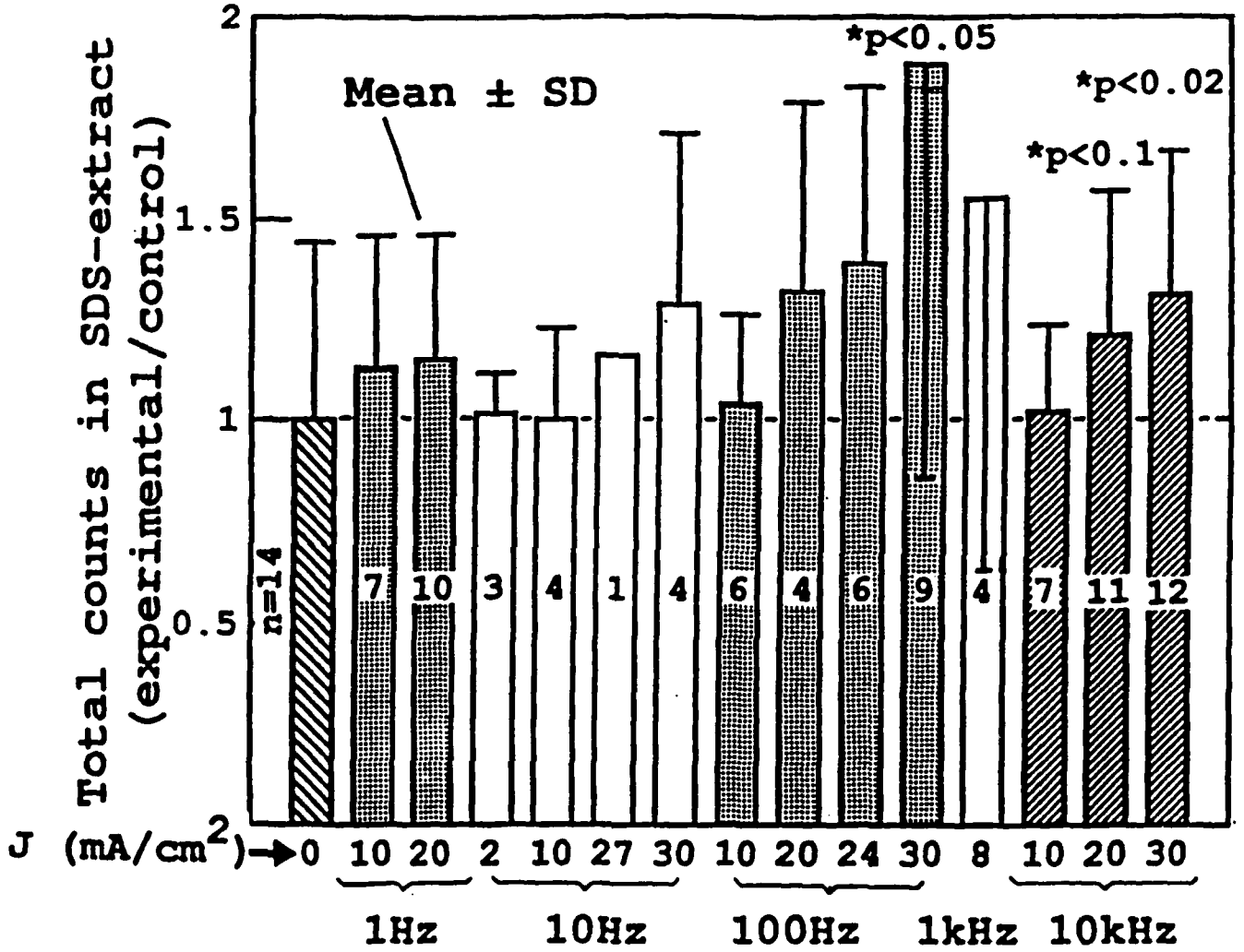

Figure 2. Protein synthesis versus applied current density in cartilage explants, relative to unstimulated control specimens. Data (mean +/- SD) are grouped by frequency from 1 Hz to 10 kHz. (Data from MacGinitie, L.A., Ph.D. Thesis, MIT, 1988.)

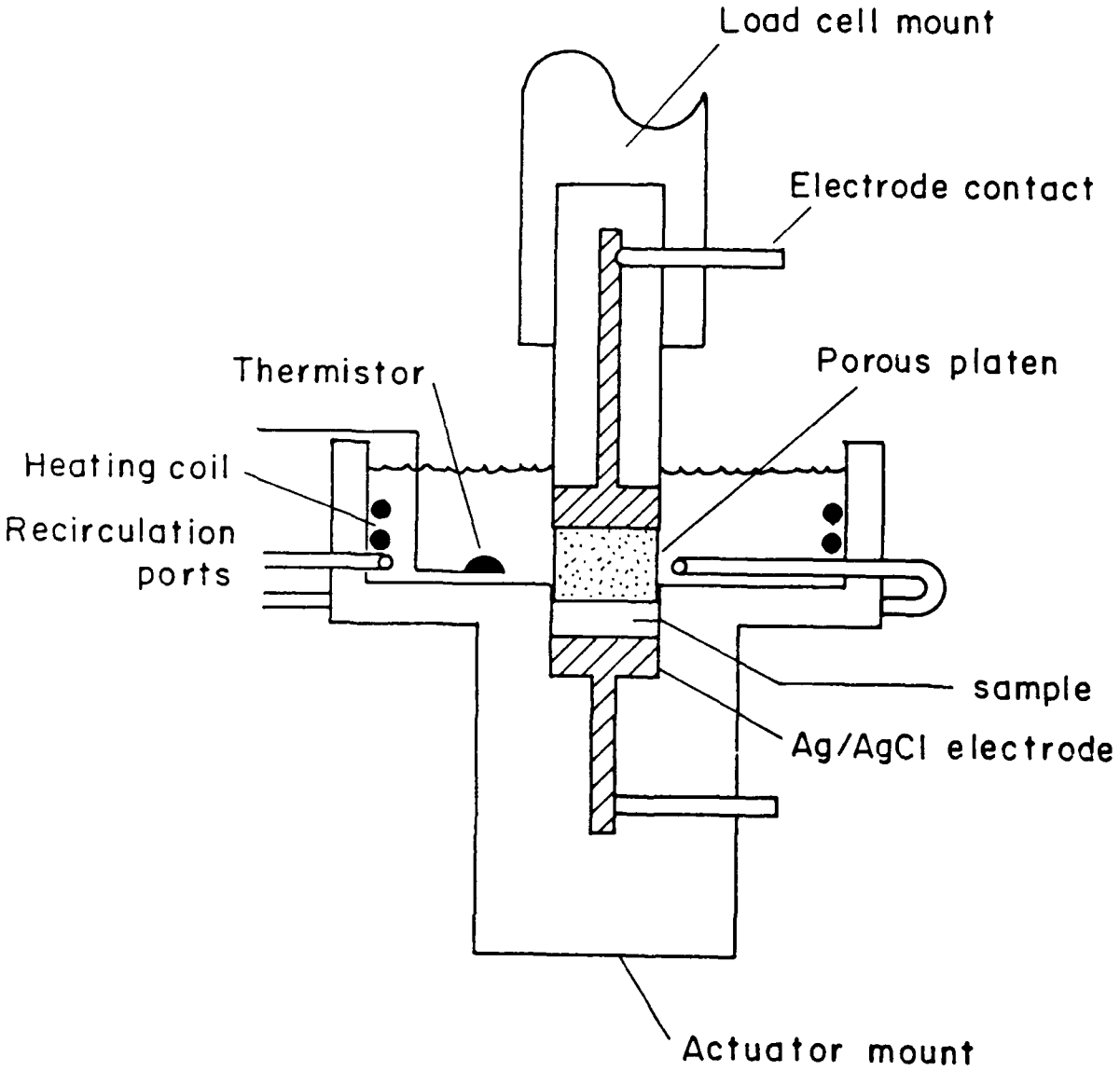

Figure 3. Schematic of test chamber used to measure the oscillatory stiffness and streaming potential of agarose/chondrocyte disks.

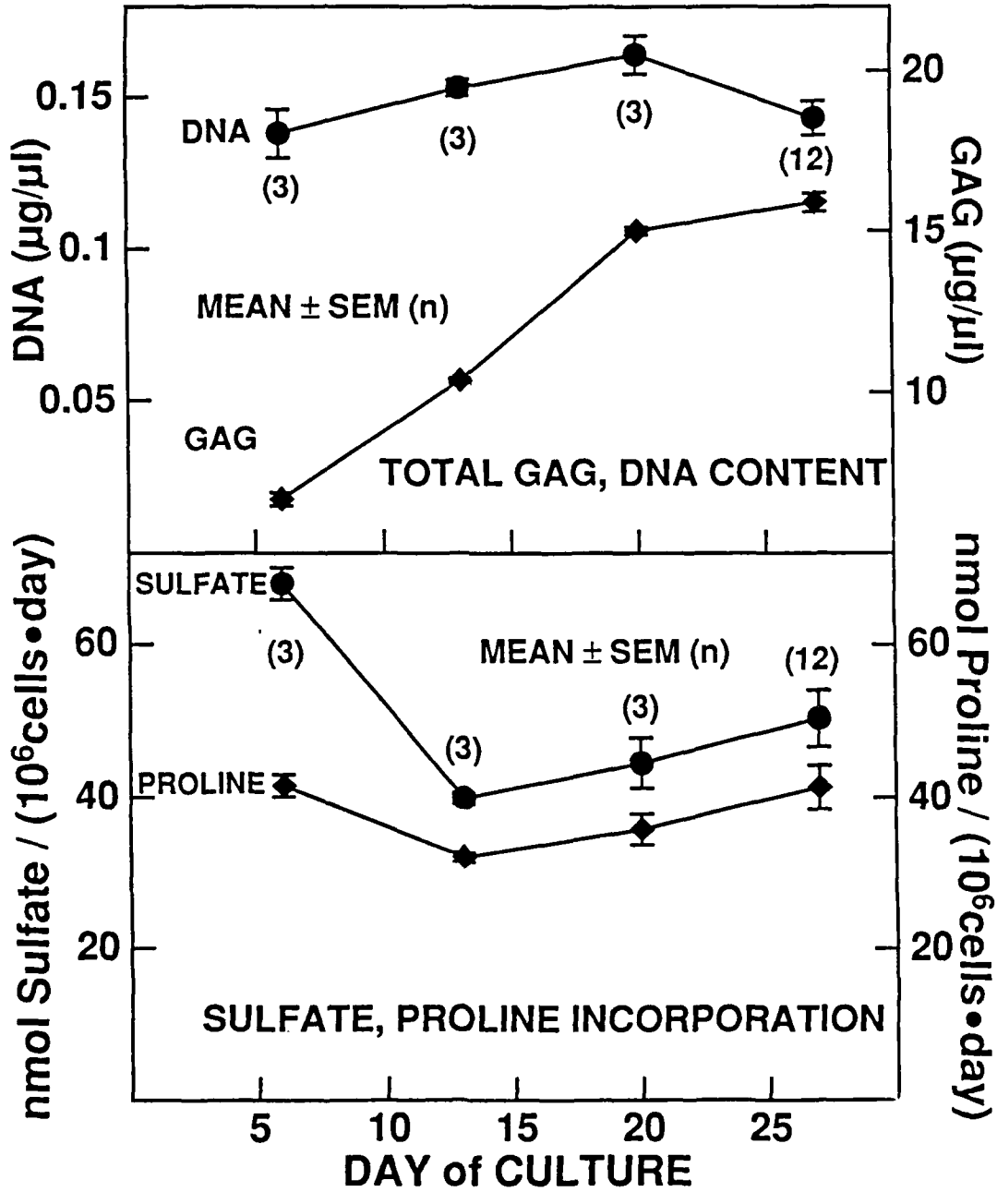

Figure 4. (A) DNA content and GAG content for chondrocyte/agarose disks versus time in culture. (B) Sulfate and proline incorporation versus time in culture, as a measure of proteoglycan and total protein synthesis, respectively.

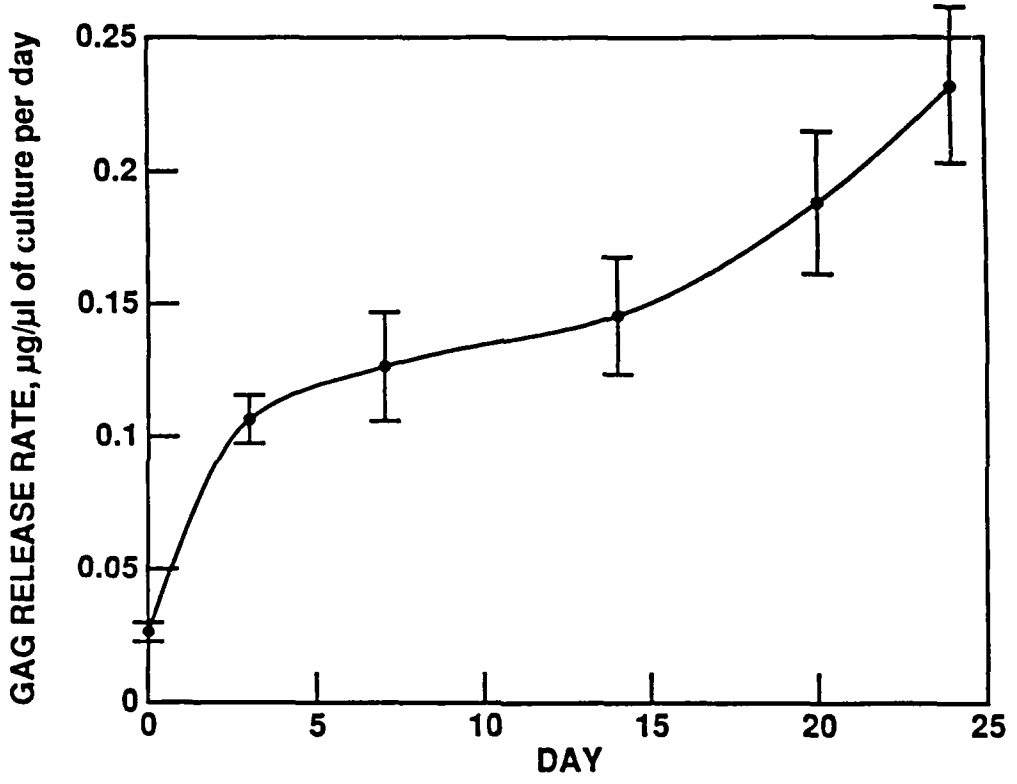

Figure 5. Rate of GAG release into the culture medium versus time in culture.

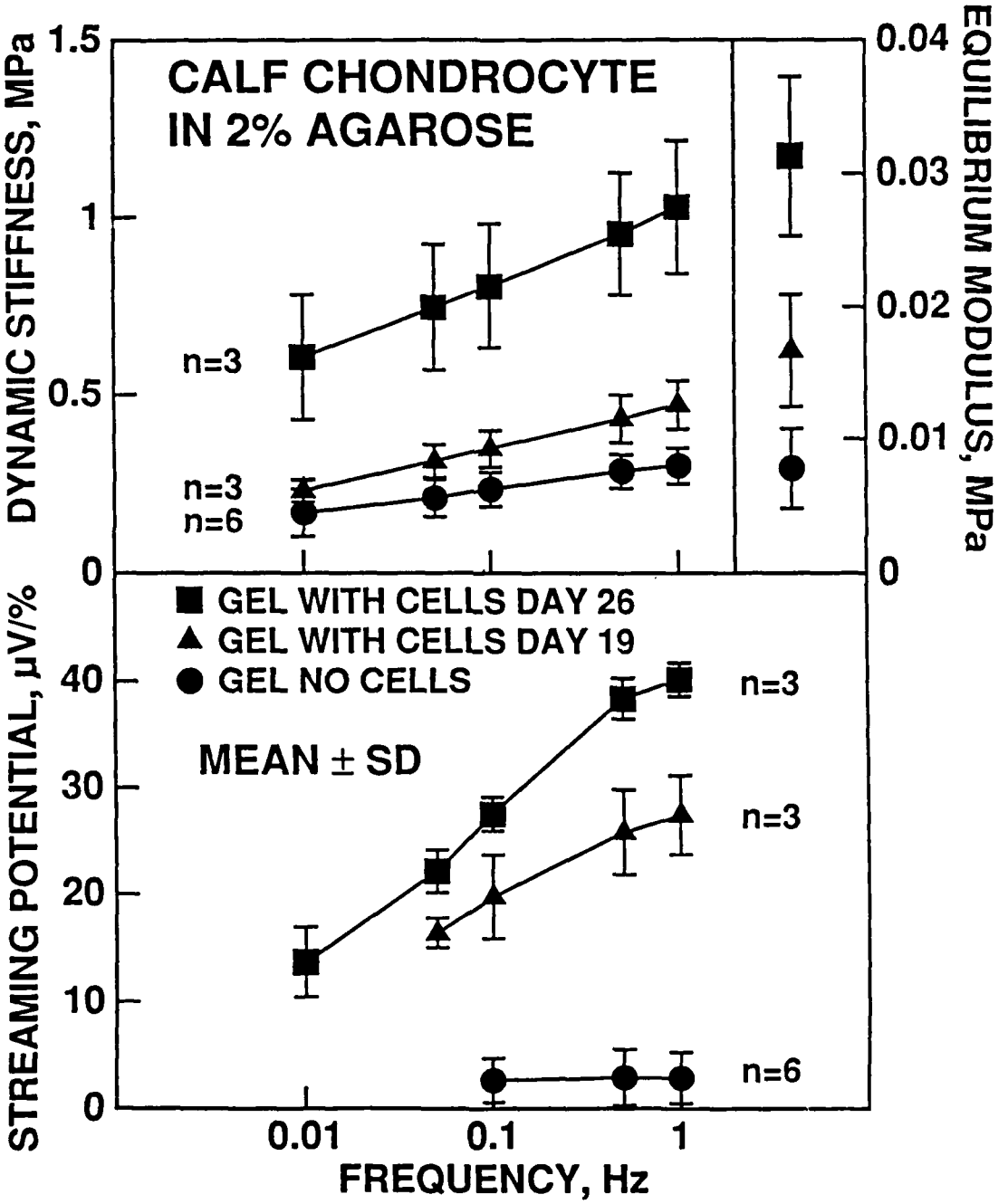

Figure 6. Dynamic stiffness and streaming potential amplitude for normal femoropatellar groove calf chondrocytes in agarose disks removed from culture on day 19 and 26 and measured versus frequency, compared to agarose disks with no cells. The equilibrium modulus of these same specimens is also shown.

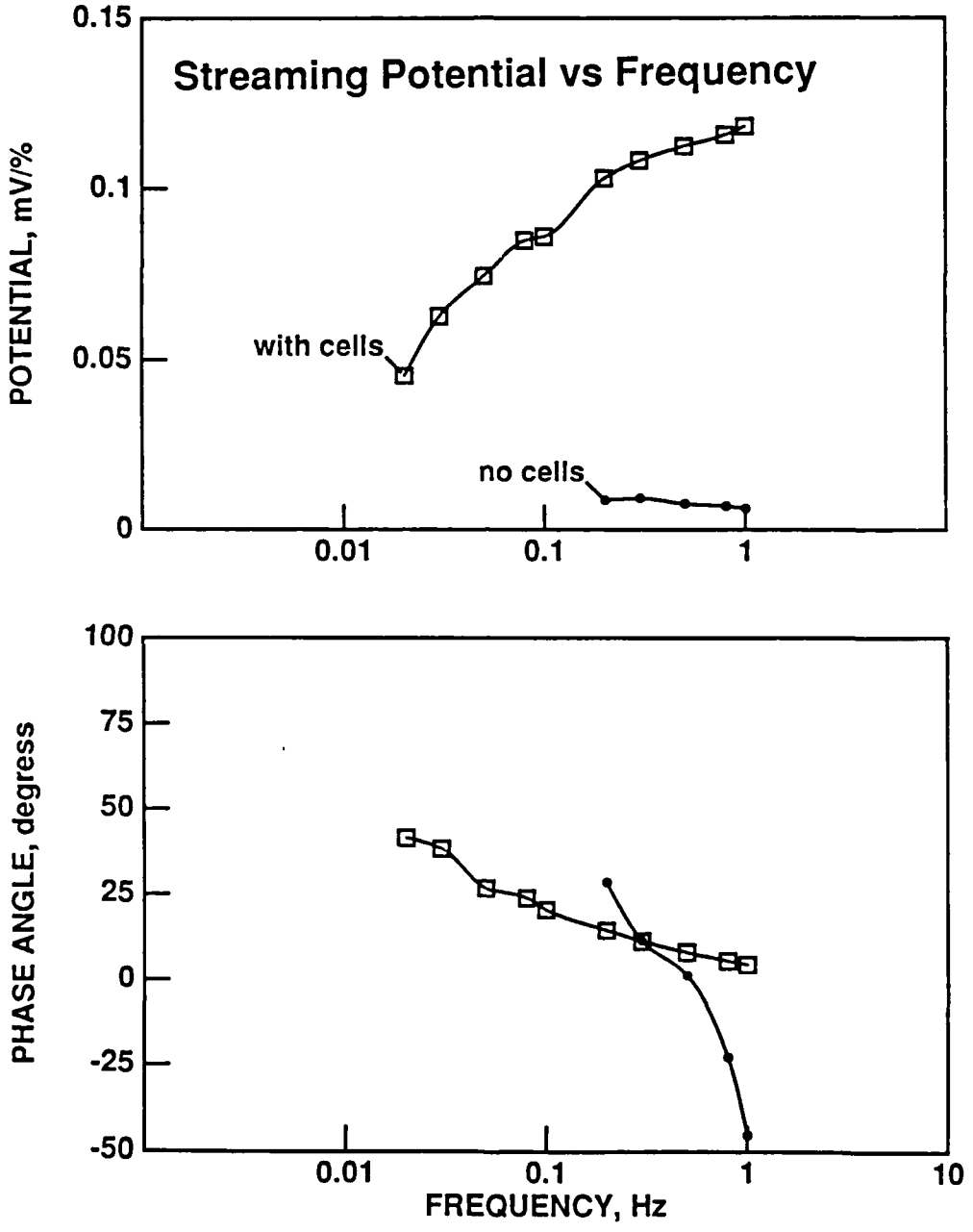

Figure 7. Streaming potential amplitude and phase angle for agarose disks containing calf metacarpal phalangeal chondrocytes versus frequency, at six weeks in culture.

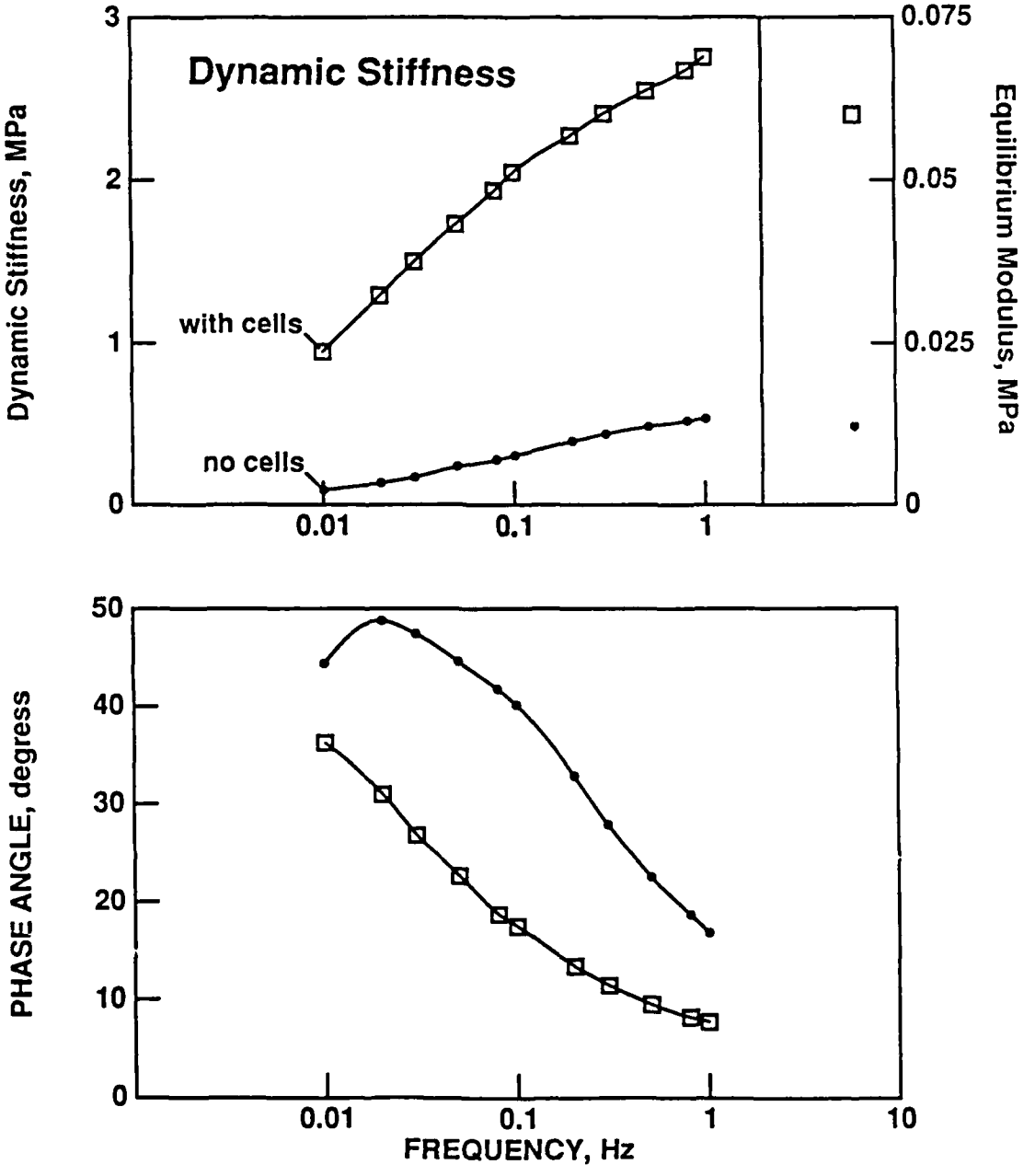

Figure 8. Dynamic stiffness amplitude and phase angle for the same specimens as Figure 7.

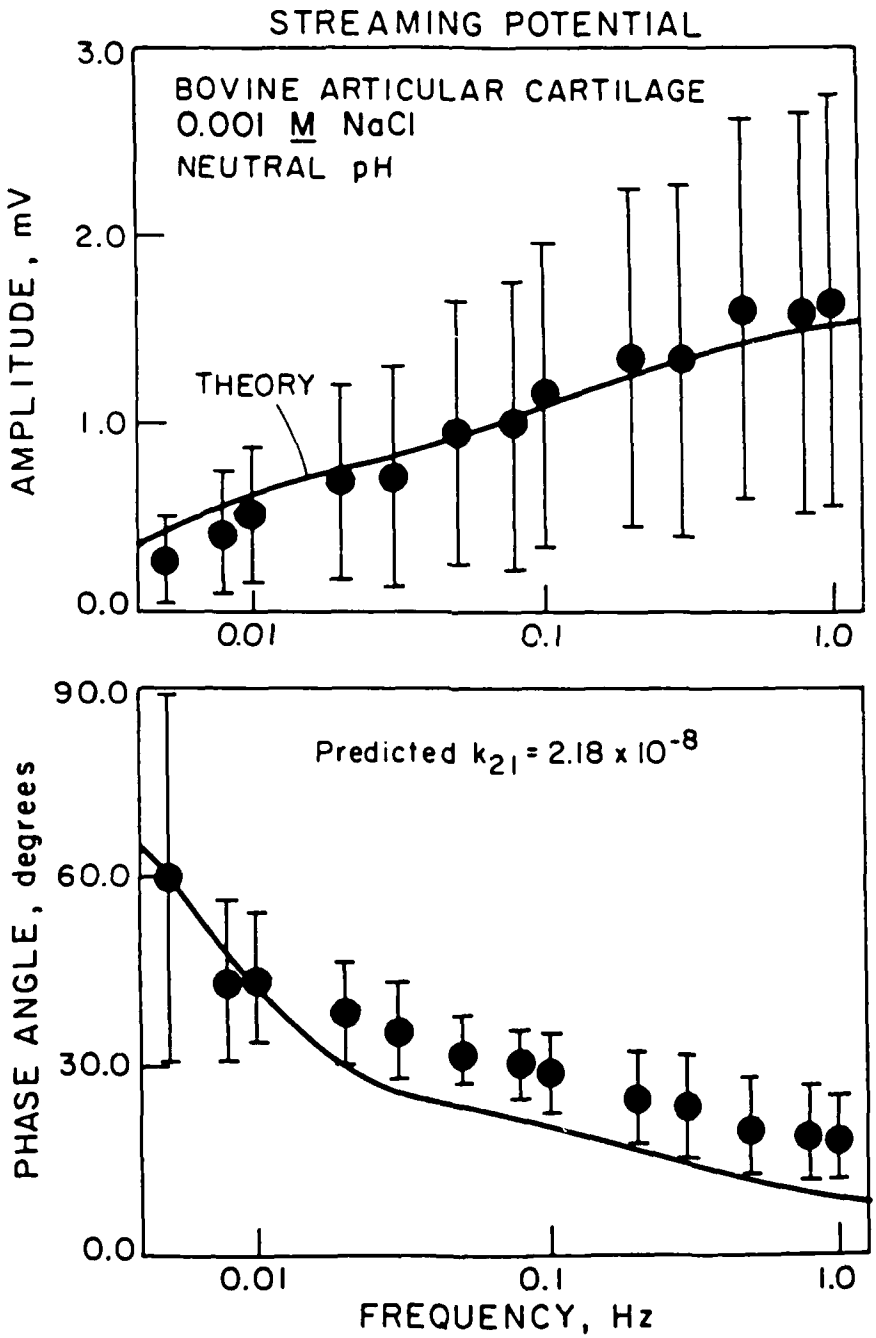

Figure 9. Streaming potential amplitude and phase angle for cylindrical disks of intact, adult bovine femoropatellar groove cartilage, for comparison with the chondrocyte/agarose data of Figures 6 and 7.

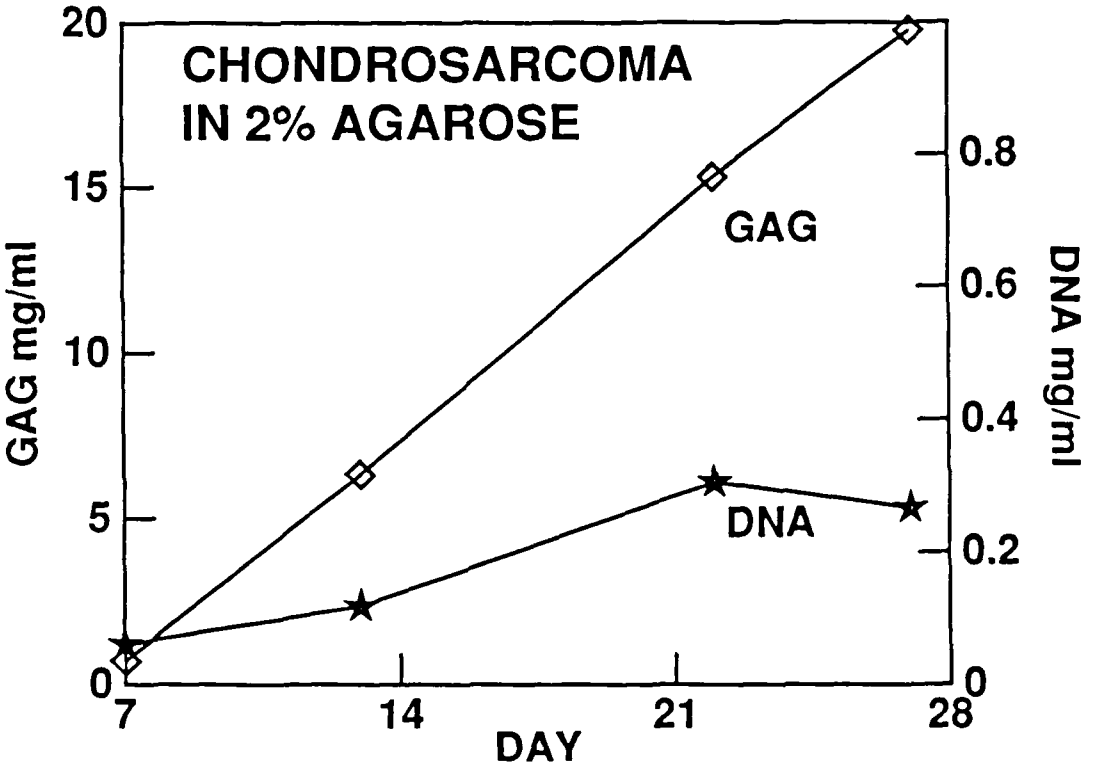

Figure 10. DNA content and GAG content versus time in culture for 2% agarose disks containing Swarm rat chondrosarcoma cells.

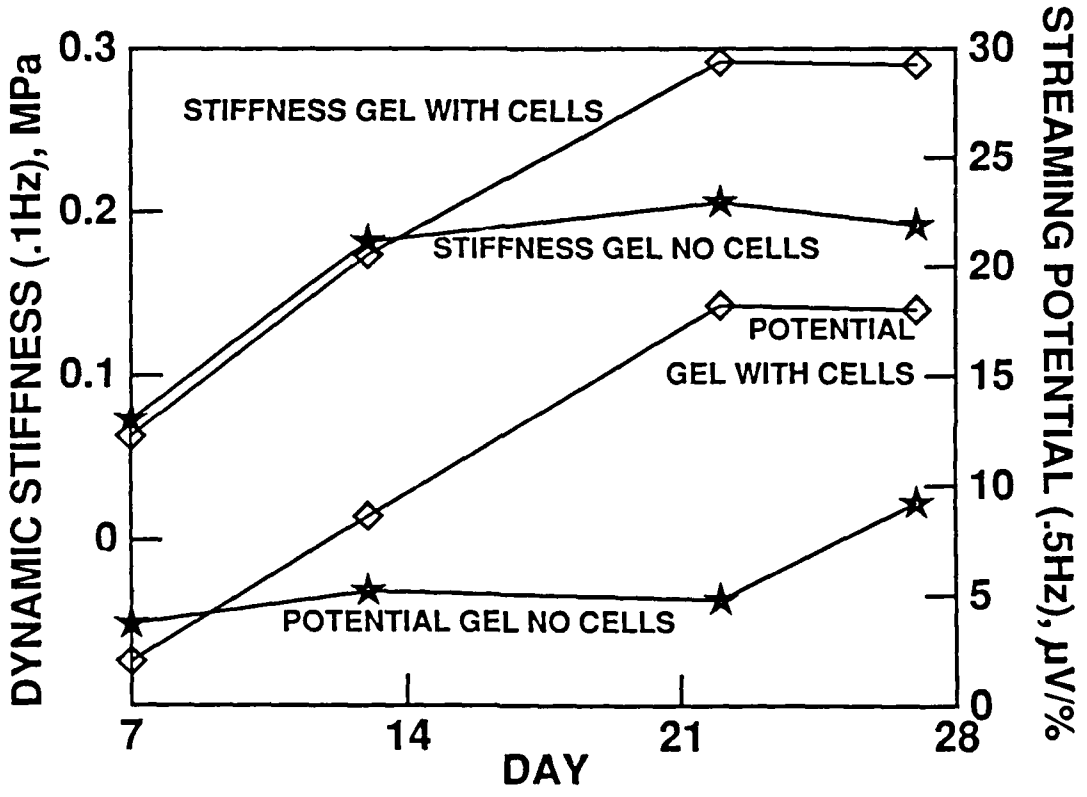

Figure 11. Dynamic stiffness and streaming potential amplitude versus time in culture for the same specimens as in Figure 10.

APPENDIX

An assay to measure the total DNA content of chondrocytes in cell-laden agarose disks was developed during this project period. Such an assay is essential to quantify chondrocyte proliferation in agarose culture, and to normalize biosynthetic and biochemical parameters to a per-cell basis.

The resulting DNA assay is based on a related method using the Hoechst 33258 dye to quantify DNA in media [27], which we recently extended in our laboratory for the purpose of measuring DNA in intact specimens of cartilage [26]. However, several problems arose when we attempted to use the cartilage DNA assay for *agarose gels* because of the presence of the agarose. Therefore, artifacts associated with the agarose had to be identified and eliminated.

The finalized DNA assay is based on a simple two-step fluorometric procedure utilizing the bisbenzimidazole dye Hoechst 33258. Test specimens in our experiments are typically cylindrical disks. After a particular experimental procedure, the agarose disks are washed and ultimately digested with papain. Because the agarose remains a gel below 65°C temperature, samples had to be heated to 70°C for 20-60 min in order to melt the gel prior to fluorometric analysis. This was found to be an essential step. After aliquots were placed in cuvettes for measurement, a period of time was required for settling. Since agarose itself contributes to the total measured fluorescence, a control study was performed to compare the fluorescence enhancement of agarose with and without chondrocytes.

Table A-1 gives a summary for the DNA assay protocol that has been developed. Figure A-1 compares the fluorescence enhancement associated with media alone and 3-mm-diameter agarose disks (without cells) equilibrated in the same media. Data are reported as DNA equivalents (μg DNA) defined by independent measurements using DNA standards in media. Figure A-1 constitutes the blank curve that is now used to subtract the fluorescence due to agarose alone from that associated with cell-laden gel disks.

Table A-1. DNA ASSAY FOR 3 mm AGAROSE/CHONDROCYTE DISKS

1) After labeling, the 3 mm disks were washed 4 times with DMEM(+), (i.e., 4 x 1.5 ml x 15 min, per 3-4 disks).

2) The disks were blotted on sterile filter paper and weighed wet inside 2 ml cryovials (and kept sterile until after papain digestion).

3) The disks (1, 3, or 4 per vial) were lyophilized in the cryovials.

4) To each sample, 1 ml of papain solution (125 µg/ml in 0.1 M sodium phosphate, 5 mM Na2-EDTA, 5 mM Cysteine-HCL, pH 6.0) was added and digested @ 60°C, 16-24 hours. (Samples were kept frozen until assayed).

5) Just before assay, the samples were heated @ 70°C for 20-60 min to melt the agarose (The melting temperature for FMC SeaPlaque agarose is 65°C.) and were immediately vortexed and allowed to cool to room temperature.

6) An aliquot of each sample was placed in an acrylic cuvet (100 µl in duplicate) and 2 ml of Hoechst 33258 dye solution was added. [see: Kim YJ, et al. Anal Biochem., 174: 168-176, 1988]

7) All the samples were mixed by repeated inversion (in cuvets with a parafilm cover), and let to settle in the dark for 1-2 hours.

8) The samples were read in a SPF 500C spectrofluorometer (SLM instruments) with excitation @ 365 nm and emission @ 458 nm (bandpass of 5 and 10 nm respectively).

9) The amount of DNA in the samples was calculated by comparison to a standard curve of known standards (treated in the same way, and using the dye as a blank) done at the time of assay. The reading due to just the agarose itself was subtracted out using a standard curve generated with blank agarose disks (i.e., disks of gel without cells).

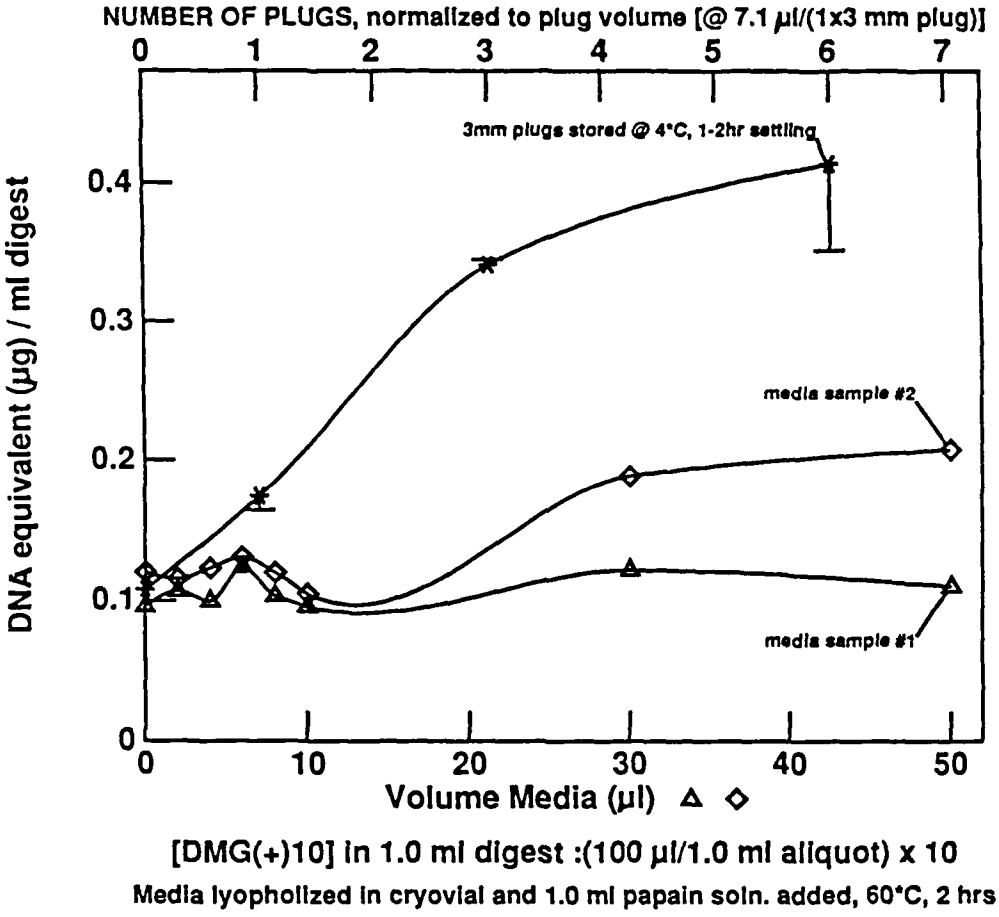

Figure A-1. Fluorescence enhancement due to media alone versus agarose disks (with no cells) equilibrated in the same media.

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